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Membrane permeability, a pivotal function involved in antibiotic resistance and virulence in *Enterobacter aerogenes* clinical isolates

J.-P. Lavigne[1]*, A. Sotto[2], M.-H. Nicolas-Chanoine[3,4], N. Bouziges[2,5], G. Bourg[2], A. Davin-Regli[1] and J.-M. Pagès[1]

1) *UMR-MD-1, Facultés de Médecine et de Pharmacie, IFR 88, Université de la Méditerranée, Marseille,* 2) *Institut National de la Santé et de la Recherche Médicale, U1047, Université Montpellier 1, UFR de Médecine, Nîmes,* 3) *Service de Microbiologie, Hôpital AP-HP Beaujon, Clichy,* 4) *Institut National de la Santé et de la Recherche Médicale, U773 Centre de Recherche Biomédicale Bichat-Beaujon, Université Paris 7, Faculté de Médecine D. Diderot, Paris and* 5) *Laboratoire de Bactériologie, Groupe Hospitalo-Universitaire de Carémeau, Nîmes, France*

Abstract

Imipenem-susceptible *E. aerogenes* isolates exhibiting extended spectrum β-lactamases, target mutations and a basal efflux expression, were identified in five patients. After imipenem treatment, imipenem-intermediate susceptible (IMI-I) or resistant (IMI-R) isolates emerged in these patients. Alteration in porin synthesis and increase in efflux expression were observed in the IMI-I isolates whereas complete loss of the porins, LPS alteration and efflux overexpression were observed in the IMI-R isolates. Bacterial virulence of the strains was investigated by the *Caenorhabditis elegans* model. The IMI-R isolates were shown to be significantly less virulent than the IMI-susceptible or IMI-I isolates. The pleiotropic membrane alteration and its associated fitness burden exhibited by *E. aerogenes* isolates influence their antibiotic resistance and their virulence behaviour. These findings highlight the balance between the low permeability-related resistance and virulence and their relationships with the treatment of resistant pathogens.

Keywords: *Caenorhabditis elegans*, efflux pumps, *Enterobacter aerogenes*, membrane permeability, multidrug resistance, porins, virulence
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Corresponding author: J.-M. Pagès, UMR-MD1, Transporteurs Membranaires, Chimiorésistance et Drug Design, Faculté de Médecine, IFR 88, 27 Bd Jean Moulin, 13385 Marseille cedex 05, France
E-mail: jean-marie.pages@univmed.fr

*Present address: INSERMU1047, Université Montpellier 1, UFR de Médecine, Nîmes, France.

Introduction

Enterobacter aerogenes has been reported since the 1990s as being increasingly resistant to various antibiotic families, resulting in multidrug-resistant (MDR) isolates [1]. The mechanism of resistance to β-lactams mostly consists of enzymatic degradation by the chromosomal cephalosporinase and plasmid-mediated broad spectrum β-lactamases [2]. The mechanical barrier, *via* the change of membrane permeability and efflux pump expression, also contributes to the increased level of resistance to β-lactams, including carbapenems, and to other antibiotic families (fluoroquinolones and chloramphenicol) [1,3,4].

After treatment including imipenem, the collected *E. aerogenes* isolates were devoid of porin and resistant to β-lactams. When the antibiotic treatment was removed, the imipenem susceptibility and the expression of porins were recovered [5,6]. This suggests the involvement of a regulator controlling their expression and a pivotal role of these outer membrane proteins in bacterial survival in the patient body, as recently mentioned for *Salmonella enterica*, *Klebsiella pneumoniae* and *Escherichia coli* strains [1,3,4]. Consequently, the modification of outer membrane permeability might have some adverse effect on the fitness and virulence level in these MDR clinical isolates. Very few internal porin mutations altering the function of the channel have been reported in clinical isolates of *Enterobacteriaceae*, suggesting that the alteration of porin might seriously disadvantage bacterial fitness [4]. Several studies reported the involvement of enterobacterial membrane transporters, including porins and efflux pumps, in modulating different processes related to colonization/virulence and

bacterial adaptation to host conditions [7–12]. This raises the question of the cost of the accumulation of membrane-associated resistance mechanisms for bacterial fitness during antibiotic chemotherapy and its consequence for bacterial capacity during the colonization process.

The aim of this work was (i) to characterize the dynamic and the impact of the membrane modifications in imipenem-resistant strains isolated during antibiotic treatment of infection caused by original susceptible *E. aerogenes* strains, (ii) to evaluate the potential virulence of these strains in the *Caenorhabditis elegans* model and (iii) to evaluate a possible link between the cost associated with the involved resistance mechanisms and the bacterial adaptation and virulence.

Materials and Methods

Bacterial collection

A prospective surveillance programme for resistant bacteria was initiated in Nîmes University Hospital. All *E. aerogenes* resistant to broad-spectrum cephalosporins (BSC) were selected. Specimens were regularly sampled from patients harbouring these strains to detect the emergence of imipenem-resistant (IMI-R) isolates during the period following the start of the imipenem treatment. If an intermediate susceptible (IMI-I) or an IMI-R isolate was identified, the strain was stocked.

Bacteria identification and antimicrobial susceptibility testing

The genus and species were determined biochemically with the Vitek 2-AST N017 identification card (bioMérieux, Marcy-l'Etoile, France). Antibiotic susceptibility was tested by using the Vitek 2 GNS-F7 card (bioMérieux).

MICs of imipenem, cefepime, chloramphenicol, ofloxacin, tetracycline and polymyxin B were determined by a standard broth dilution method in triplicate [6,13]. The efflux pump inhibitor phenylalanyl arginine β-naphthylamide (PAβN) (Sigma-Aldrich, St-Quentin Fallavier, France) was used at 26.3 mg/L, for which no intrinsic effect has been observed [14]. EDTA (0.5 mM) was used to evaluate the involvement of outer membrane alteration in the polymyxin B resistance [3,15,16]. *E. aerogenes* strain ATCC 13048 and EA27 clinical isolate, which overexpressed the AcrAB efflux system, were used as controls [6,17]. Isolates were classified as susceptible, intermediate susceptible or resistant to the antibiotics according to the recommendations of the Antibiotic Susceptibility Testing Committee of the French Society for Microbiology (http://www.sfm.asso.fr/nouv/general.php?pa=2). Enzymatic production was checked by the double-disk synergy test using MH agar with 250 mg/L cloxacillin (http://www.eucast.org/clinical_breakpoints/). AmpC hyperproduction was evaluated by measuring MICs of ceftazidime, cefepime and imipenem with the E-test method using MH agar without and with 250 mg/L cloxacillin.

Characterization of β-lactamase-encoding genes and fluoroquinolone resistance

The bla_{TEM} gene was detected by PCR and identified by sequencing the PCR products as previously reported [18]. *gyrA*, *gyrB*, *parC, parE, qnrA, qnrB* and *qnrS* were screened by PCR and PCR products were sequenced to detect mutations as previously described [19].

PFGE analysis

Macrorestriction analysis of chromosomal DNA was performed using PFGE according to published procedures after *Xba*I restriction (New England Biolabs France, Evry, France) using the CHEF system (Bio-Rad SA, Ivry-sur-Seine, France) [20]. The PFGE patterns were analysed with Gel compar computer software (Applied Math, Kortrijk, Belgium) and an unweighted-pair group method with the Dice coefficient of similarity. Isolates were considered to be within a cluster if the coefficient of similarity was >80%. The different isolates were compared with the EAI_A strain belonging to the French *E. aerogenes* clone [20].

SDS-polyacrylamide gel electrophoresis and outer membrane proteins identification

Exponential-phase bacteria in LB broth were pelleted and solubilized [6,9]. Samples (bacterial amount corresponding to 0.02 optical density units at 600 nm) were loaded onto SDS-polyacrylamide gels (10%, 14% polyacrylamide, 0.1% SDS), then electrotransferred to nitrocellulose membranes. The immunodetections were carried out as previously described with polyclonal antibodies directed against outer membrane proteins (Omp35, Omp36, OmpX and OmpA) and against the efflux pump components (AcrA and TolC) [13,21]. *E aerogenes* strains ATCC 13048, EAEP289 (a derivative of strain EA27 exhibiting norfloxacin efflux), EAEP294 and EAEP298 (EAEP289 *acrA* or *tolC* derivatives, respectively) were used as controls.

The method for extracting outer membranes was described previously [22]. The band corresponding to a new major protein was excised from the SDS-polyacrylamide gels and sequenced by mass spectrometry. Determination of the degrees of identity and similarity with known proteins was carried out using BLASTX and BLASTP and FASTA from the Genetics Computer Group (GCG, Princeton, NJ, USA) [23].

Detection of high-pathogenicity island (HPI)

The presence of the HPI was determined by PCR analysis as previously described [24].

Nematode killing assay

The *C. elegans* model has been developed to study host-pathogen interactions and to identify the basic pathways well conserved during evolution that are linked to microbial pathogenesis. In this test, the studied bacteria are presented as food to the nematodes instead of *E. coli* strain OP50, an avirulent strain, which is their usual food in the laboratory. Ingestion of the bacteria by the worms results in an infection and ultimately death of the worms. The time required by the studied bacteria to kill the worms compared with the life duration observed when the worms are fed with *E. coli* strain OP50, is an indirect marker of virulence potential of the bacteria. The *C. elegans* infection assay was carried out by using the Fer-15 mutant line as previously published [25]. At least two replicates repeated six times were performed for each selected clone. Lethal time 50% (LT50) and lethal time 100% (LT100) corresponded to time (in days) required to kill 50% and 100% of the nematode population, respectively. *E. aerogenes* strains ATCC13048 and CM64 (exhibiting an active efflux) were used as reference.

The number of bacteria within the *C. elegans* digestive tract was obtained as previously described [25]. *C. elegans* were picked at 72 h, and the surface bacteria were removed by washing the worms twice in 4 μL drops of M9 medium on a BHI agar plate containing 25 mg/L gentamicin. The nematodes were placed in a 1.5-mL Eppendorf tube containing 20 μL of M9 medium with 1% Triton X-100 and were mechanically disrupted. The volume was 50 μL (M9 medium containing 1% Triton X-100), which was diluted and plated on BHI agar containing 50 mg/L ampicillin. Three replicates were performed for each bacterial combination.

To compare the entire survival curves in nematode killing assays, Cox regression was used. To perform pairwise comparison between two different strains, we used a log rank test. The analysis was carried out using SPSS 6.1.1 (SPSS Inc., Chicago, IL, USA).

Results

Strains and antibiotic susceptibility

During the studied period (2 years), five patients (A–E) presented imipenem-susceptible *E. aerogenes* strains, then intermediate or resistant (I or R) isolates. The strains exhibited PFGE profiles with more than 90% similarity between each other and with strain EAI_A corresponding to the French *E. aerogenes* clone (data not shown). The isolates IMI-S, IMI-I or/and IMI-R from a given patient presented an identical profile (100% similarity, data not shown).

The antibiotic susceptibilities are presented in Table 1. Among the IMI-S isolates (S), all were resistant to cefepime, chloramphenicol and ofloxacin, and 4/5 to tetracycline. No isolate was resistant to polymyxin B. For IMI-I and IMI-R isolates, the distribution of resistance percentages was quite similar to those of IMI-S except for polymyxin.

The PAβN addition reduced the MICs for ofloxacin and chloramphenicol in all strains (Table 1). Regarding tetra-

TABLE 1. Antibiotic susceptibilities of *E. aerogenes* isolates to different antibiotics with or without efflux pump inhibitor or EDTA. For each patient, the first isolate corresponds to the susceptible strain isolated before IMI treatment and the others correspond to the intermediate- and resistance-IMI strains

		MIC (mg/mL) (with efflux pump inhibitor[a]) [with EDTA[b]]					
Patient	**Strains**	**CHL**	**TET**	**OFL**	**IMI**	**FEP**	**PMB**
A	NEA39541S	>512 (64)	64 (32)	64 (16)	≤0.25 (≤0.25)	32 (32)	0.5
	NEA354871I	512 (64)	128 (32)	64 (16)	8 (4)	>32 (32)	0.5
B	NEA567234S	32 (≤4)	≤2 (≤1)	32 (2)	2 (0.5)	8 (4)	1
	NEA575169I	128 (64)	16 (8)	32 (2)	4 (0.25)	8 (8)	1
C	NEA31004S	512 (64)	8 (8)	64 (8)	1 (0.25)	32 (32)	1
	NEA23214R	>512 (64)	8 (8)	64 (8)	32 (8)	>32 (>32)	2
D	NEA48271S	512 (64)	8 (8)	128 (8)	1 (0.5)	32 (32)	1
	NEA513511	256 (64)	2 (1)	64 (8)	4 (0.25)	32 (32)	1
E	NEA43735R	512 (64)	4 (4)	64 (16)	16 (0.25)	>32 (32)	2
	NEA709472S	32 (32)	128 (≤1)	≤4 (≤2)	0.5 (0.25)	32 (32)	1
	NEA734521I	512 (64)	128 (32)	64 (4)	8 (2)	32(32)	1
	$NEA736500_{BC}R$	512 (16)	128 (8)	128 (8)	16 (4)	32 (16)	2
	$NEA736500_{SC}R$	256 (64)	128 (8)	64 (4)	32 (4)	64 (32)	128 [2]
Reference strains	ATCC13048	4 (2)	≤4	≤4	1	≤8	1
	EA27	512 (64)	8	128	8	>32	nd

CHL, chloramphenicol; TET, tetracyclines; OFL, ofloxacin; IMI, imipenem; FEP, cefepime; PMB, polymyxin B; S, susceptible to imipenem; I, intermediately susceptible to imipenem; R, resistant to imipenem.
$NEA736500_{BC}R$ is an isolate with normal growth (BC, big colonies) and $NEA736500_{SC}R$ is an isolate with slow growth (SC, small colonies).
[a]PABN used at 26.3 mg/L.
[b]EDTA used at 0.5 mM.
[c]ATCC 13048 strain with a normal level of AcrAB efflux system; EA27, clinical isolate with an over-expression of AcrAB efflux system.

cycline susceptibility, the patient E isolates were strongly sensitized by PAβN. One isolate (NEA736500$_{SC}$R) was resistant to polymyxin B. In the presence of EDTA, which chelates polycations associated with LPS [59,60], polymyxin MIC decreased from 128 mg/L to 2 mg/L (Table 1).

All the IMI-S and IMI-I/R-resistant isolates harboured the broad-spectrum β-lactamase TEM-24 and AmpC-enzyme. For AmpC, no significant variation was detected between the MICs obtained with E-tests and those obtained by the dilution method. The decrease in ceftazidime MICs with cloxacillin confirmed the over-production of AmpC (data not shown).

None of the tested *qnr* genes were detected. GyrA (Ile83-Ser, numbering according to *E. coli*) and ParC (Ser80Ile) modifications were identified in all the isolates supporting the significant quinolone MICs in addition to PAβN-sensitive efflux.

Membrane transporters: detection of pump components and outer membrane proteins

The signals obtained from RT-PCR analysis did not systematically correlate with the functional assembly of bacterial membrane proteins [13]. Consequently, the analyses were carried out by staining the membrane protein fractions and by performing western blot analysis [13,14].

For IMI-S isolates, as indicated in Table 2 and Fig. 1 for the strains of patient E, the AcrA and TolC signal observed was slightly higher than that of the reference strain ATCC 13048. Outer membrane proteins OmpA and OmpX were detected at the same expression level (Table 2). For Omp35 and Omp36, all IMI-S isolates exhibited a positive signal (Table 2).

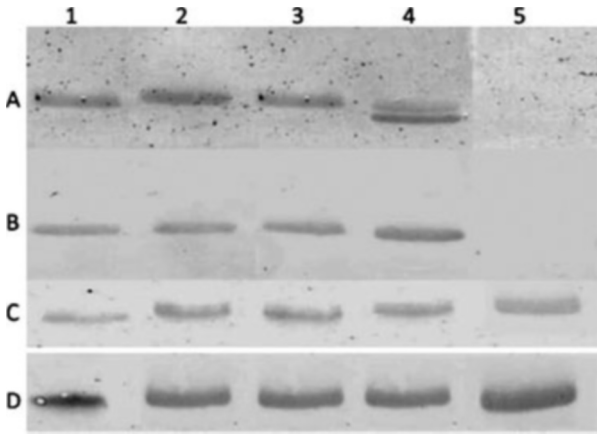

FIG. 1 Detection of porins and efflux pump components in *E. aerogenes* strains isolated in Patient E. Lane 1, ATCC13048; lane 2–5, strains NEA709472S, NEA734521I, NEA736500$_{BC}$R and NEA736500$_{SC}$R. A, Omp35 profile; B, Omp36 profile; C, AcrA profile; D, TolC profile.

For IMI-I/R strains, an increased level of AcrA and TolC, similar to that of strain EA27, was observed in all strains except for strain NEA736500$_{SC}$R (Patient E). This isolate exhibited the highest signal for TolC (Fig. 1 and Table 2). No change of OmpA and OmpX expression level was detected (Table 2). For the Omp35 signal three profiles can be observed: (i) an Omp35 expression in IMI-I strains for which IMI MICs were 4–8 mg/L (n = 4); (ii) a decreased Omp35 level and the emergence of a new band for NEA736500$_{BC}$R for strains having an IMI MIC 16 mg/L (n = 1); and (iii) no expression of Omp35 in IMI-R strains, IMI MICs between 16–32 mg/L (n = 3). Similar results were obtained with

TABLE 2. Evolution of outer membrane protein, efflux pump components, LPS and bacterial growth for the different *E. aerogenes* strains isolated at baseline and during the imipenem treatment period

Patient	Strain	AcrA	TolC	OmpA	OmpX	Omp36	Omp35	OmpA-like	LPS	Bacterial growth: $OD_{600\ nm}$ at 24 h
A	NEA39541S	+	++	++	+	++	++	–	–	≥2
	NEA354871I	++	++	++	+	++	+	–	–	≥2
B	NEA567234S	+	++	++	+	++	++	–	–	≥2
	NEA575169I	++	++	++	+	++	++	–	–	≥2
C	NEA31004S	+	++	++	+	++	++	–	–	≥2
	NEA23214R	++	+++	++	+	–	–	–	–	≥2
D	NEA48271S	+	+	++	+	++	++	–	–	≥2
	NEA513511	++	+	++	+	++	+	–	–	≥2
E	NEA43735R	++	++	++	+	–	–	–	–	≥2
	NEA709472S	+	+	++	+	++	++	–	–	≥2
	NEA734521I	+	++	++	+	++	++	–	–	≥2
	NEA736500$_{BC}$R	++	++	++	+	+	+	+	–	≥2
	NEA736500$_{SC}$R	++	+++	++	++	–	–	–	++	1.110
ATCC13048		+	+	+	+	+	+	–	–	≥2
EA27		++	++	++	++	–	–	–	–	≥2

For Omp proteins, –corresponds to absence of signal, + to a slight and ++ to a high porin expression. For AcrA and TolC, –corresponds to absence of signal, + to a basal level (ATCC strain) and ++ to a high efflux pump expression.
For LPS, –corresponds to PMB-susceptible strains (no LPS alteration), ++ to PMB-resistant strains (a high LPS alteration). For bacterial growth, +/– corresponds to a very slow bacterial growth and ++ to a normal bacterial growth.
S, susceptible; I, intermediate susceptible; R, resistant.

antibodies directed against Omp36 (Table 2). The new band, observed in isolate NEA736500$_{BC}$R, was identified by mass spectrometry to an OmpA-like protein.

Virulence of *E. aerogenes* isolates and bacterial growth

The HPI virulence factor-encoding genes were detected in all isolates. The *C. elegans* model is well described for the study of bacterial factors involved in infection and colonization [26,27]. In the nematode killing assay, all IMI-S *E. aerogenes* isolates were significantly more virulent (LT50 3.5–4.3 ± 0.5 days; LT100 7.8–8.3 ± 0.9 days) than both IMI-I and IMI-R isolates (LT50 5.1–6.9 ± 0.8 days; LT100 10.3–13.5 ± 0.7 days) ($p < 0.001$) (Table 3). The IMI-I isolates were significantly more virulent than the IMI-R isolates (LT100 10.3–10.8 ± 0.8 days vs. 12.0–13.5 ± 0.9 days, respectively) ($p < 0.001$). The IMI-S isolates presented a similar level of virulence to strain ATCC13048 (LT100$_{ATCC13048}$ 8.4 ± 0.6 days). Strain NECS736500$_{SC}$R, which displayed no virulence, similarly to the avirulent *E. coli* OP50 strain (Table 3), was significantly less virulent than other tested IMI-R isolates.

As shown in Table 2, the bacteria had a similar *in vitro* growth independently of the IMI susceptibility phenotype except for one strain, NEA736500$_{sc}$R, presenting a very slow growth curve (data not shown). For the later strain, small colonies were observed on agar medium and IMI and polymixin B MICs were the highest for this isolate (Table 1). The number of *E. aerogenes* CFU within the nematode gut varied around 10^6 bacteria per worm for each strain 72 h after ingestion without statistical difference (Table 4), confirming the presence of *E. aerogenes* isolates in the *C. elegans* intestine.

TABLE 4. Evaluation of CFU of clinical isolates and control strains within the *C. elegans* digestive tract

Patients	Strain	Median CFU [range]/ nematode after 72 h
A	NEA39541S	1.5×10^6 [$0.9–1.7 \times 10^6$]
	NEA354871I	2.9×10^6 [$2.1–3.4 \times 10^6$]
B	NEA567234S	1.2×10^6 [$0.9–2.0 \times 10^6$]
	NEA575169I	0.5×10^6 [$0.2–1.0 \times 10^6$]
C	NEA31004S	1.1×10^6 [$0.5–1.5 \times 10^6$]
	NEA23214R	5.2×10^6 [$4.0–5.7 \times 10^6$]
D	NEA48271S	7.1×10^6 [$6.2–8.2 \times 10^6$]
	NEA513511	2.1×10^6 [$1.8–2.6 \times 10^6$]
	NEA43735R	3.8×10^6 [$3.0–4.5 \times 10^6$]
E	NEA709472S	5.1×10^6 [$4.4–6.0 \times 10^6$]
	NEA734521I	2.3×10^6 [$1.6–3.1 \times 10^6$]
	NEA736500$_{BC}$R	4.2×10^6 [$3.8–4.5 \times 10^6$]
	NEA736500$_{SC}$R	1.3×10^6 [$0.6–1.5 \times 10^6$]
Reference strains	ATCC 13048	2.7×10^6 [$1.8–3.5 \times 10^6$]
	CM64	4.3×10^6 [$2.9–5.1 \times 10^6$]

CM64, ATCC 13048 derivative exhibiting an active efflux.

Discussion

This study describes for the first time a link between (i) the alteration of *E. aerogenes* membrane physiology and the impact of this on antibiotic susceptibility and (ii) the colonization capability of *E. aerogenes* in the nematodes.

Various changes in the membrane of *E. aerogenes* isolates were observed during the antibiotic treatment of patient E and we scrutinized the bacterial evolution following three main stages. In the first stage, the isolate presented a resistance to β-lactams (due to enzymatic expression) associated with resistance to other antibiotic families, notably to fluoroquinolones (efflux pump and topoisomerase mutations). In the second stage, the isolate became less susceptible to imipenem and exhibited membrane modifications with a decrease in porin

TABLE 3. *In vivo* kinetics of killing of *C. elegans* infected by *E. aerogenes* strains (Str). Comparison of LT50 and LT100 between the different strains tested. The results are representative of at least six independent trials for each group of strains. *E. coli* OP50, *E. aerogenes* ATCC13048 and CM64 represent control strains

				P					
Patients	Strains	LT50	LT100	S vs. I	S vs. R	I vs. R	Str vs. CM64	Str vs. OP50	Str vs. ATCC
A	NEA39541S	3.5 ± 0.5	7.8 ± 0.8	–	<0.001	–	–	<0.001	NS
	NEA354871R	6.0 ± 0.2	12.0 ± 0.5	–	<0.001	–	<0.001	<0.001	<0.001
B	NEA567234S	4.2 ± 0.2	8.3 ± 0.7	<0.001	–	–	<0.001	<0.001	NS
	NEA575169I	5.1 ± 0.3	10.3 ± 0.7	<0.001	–	–	<0.001	<0.001	<0.001
C	NEA31004S	4.3 ± 0.3	8.3 ± 0.7	–	<0.001	–	<0.001	<0.001	NS
	NEA23214R	6.6 ± 0.4	12.3 ± 0.7	–	<0.001	–	<0.001	<0.001	<0.001
D	NEA48271S	4.0 ± 0.2	8.0 ± 0.5	<0.001	<0.001	–	<0.001	<0.001	NS
	NEA513511	5.4 ± 0.4	10.8 ± 0.8	<0.001	–	<0.001	<0.001	<0.001	<0.001
	NEA43735R	7.1 ± 0.2	12.4 ± 0.4	–	<0.001	<0.001	<0.001	<0.001	<0.001
E	NEA709472S	4.1 ± 0.3	8.0 ± 0.5	<0.001	<0.001	–	<0.001	<0.001	NS
	NEA734521I	5.2 ± 0.2	10.8 ± 0.8	<0.001	–	<0.001	<0.001	<0.001	<0.001
	NEA736500$_{BC}$R	6.5 ± 0.5	12.1 ± 0.9	–	<0.001	<0.001	<0.001	<0.001	<0.001
	NEA736500$_{SC}$R	6.9 ± 0.2	13.5 ± 0.5	–	<0.001	<0.001	<0.001	NS	<0.001
Reference strains	OP50	8.6 ± 0.3	14.0 ± 0.5	ND	ND	ND	<0.001	ND	<0.001
	CM64	3.2 ± 0.2	6.4 ± 0.6	ND	ND	ND	ND	<0.001	<0.001
	ATCC13048	4.4 ± 0.2	8.4 ± 0.6	ND	ND	ND	<0.001	<0.001	ND

ND, not determined; NS, not significant; S, susceptible to imipenem; I, intermediately susceptible to imipenem; R, resistant to imipenem.

production and over-expression of efflux pumps. In the third stage, the isolate was resistant to imipenem and presented an over-expression of efflux pumps conjointly with loss of the main porins, and an alteration of LPS. The complete loss of the two major porins and the LPS alteration were clearly involved in strains exhibiting a high level of resistance to imipenem and polymyxin as previously suggested [5,6].

These results indicate a strong modification of the organization and physiology of the outer membrane that has a direct impact on the bacterial fitness. Indeed, the NEC736500$_{SC}$R isolate exhibiting polymyxin resistance, presented a slow growth with loss of porins and a maintained over-expression of efflux pumps. The strain severely restricts the membrane permeability, preventing any entry (e.g. antibiotics, nutrients that use the porin way), and its fitness slows down subsequently so that it only maintains elements providing bacterial survival [28].

Only a very few studies assess the virulence of MDR bacteria collected during antibiotic treatment and exhibiting various levels of susceptibility. We reported here that the MDR strains presenting the highest level of resistance to IMI-CHL-OFL-FEP were significantly less virulent than other strains. The AmpC-producing and BSC-producing isolates collected during the first stage were as virulent as strain ATCC 13048, suggesting that β-lactamase expression and bacterial virulence are non-overlapping mechanisms. An increased virulence is observed in strains harbouring an active efflux pump as previously reported [29,30]. The expression of RND efflux pump like AcrAB-TolC is a prerequisite in the early bacterial colonization step to pass the bile salt barrier or defensin activity [8,9,29,30]. Lastly, an abolished virulence in strains presenting simultaneously a loss of porins and LPS alterations is reported.

In the *C. elegans* model, the reduction of *E. aerogenes* virulence is associated with the membrane modifications involved in drug resistance. The combination of outer membrane alterations induces an important decrease in bacterial fitness and adaptation to environmental stresses. Porin and LPS organizations contribute to the physiological state involved in the mid-late/late steps of host colonization [9,11,12]. Consequently, porin deficiency and LPS modifications selected during antibiotic pressure generate a non-physiological membrane state with a fitness cost that decreases the bacterial capability for infection/colonization.

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Transparency Declaration

Nothing to declare.

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